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SHORT COMMUNICATION

Preliminary evidence supports circulating microRNAs as prognostic biomarkers for type 2 diabetes

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Summary

Background

Circulating microRNAs are emerging as potential prognostic biomarkers for the development of type 2 diabetes. However, microRNAs are also associated with complications from impaired glucose metabolism (e.g. endothelial cell function). Prior studies have not evaluated for associations between trajectories of circulating microRNAs with trajectories of fasting blood glucose over time and the responses to behavioral interventions to reduce risk. This study performed longitudinal assessment of microRNAs and fasting blood glucose and identified relationships between microRNAs and behavioral risk reduction interventions.

Methods

MicroRNAs (n = 353) were measured in subsets (n = 10, n = 8) of participants from previously completed clinical trials that studied behavioral risk reduction interventions. Fasting blood glucose trajectories were associated with changes in 45 microRNAs over 12 months.

Results

Following a 3-month physical activity and dietary intervention compared with baseline, 13 microRNAs were differentially expressed. Seven microRNAs (i.e. miR-106b, miR-20b, miR-363, miR-486, miR-532, miR-92a and miR-93) were commonly identified between the two analyses.

Conclusions

Further studies are needed to determine which microRNAs are prognostic biomarkers of risk for type 2 diabetes versus consequences of impaired glucose metabolism. Additional future directions of this research are to differentiate whether microRNAs are prognostic and/or diagnostic biomarkers for risk for type 2 diabetes and predictive biomarkers of responses to risk reduction interventions.

Keywords: biomarker, diabetes, fasting blood glucose, microRNA.

Introduction

Given the complex etiology, type 2 diabetes was recently identified as a priority for the field of precision health (1). In most people, risk for type 2 diabetes begins with insulin resistance, which is largely undetectable in clinical practice, and progresses to impaired fasting glucose (i.e. fasting blood glucose 100–125 mg dL) before a diagnosis of type 2 diabetes is made. During this prodromal window prior to the diagnosis of type 2 diabetes, harmful conditions (e.g. inflammation) can occur and go largely undetected. (2,3) Common biomarkers (i.e. fasting blood glucose and hemoglobin A1c) for type 2 diabetes are used for diagnosis and monitoring of the disease but do not identify which individuals are at greatest risk for developing the disease in the prodromal window. Newer

biomarkers that provide prognostic information about risk for type 2 diabetes have the potential to improve risk stratification.

MicroRNAs are short (i.e. 18–26 nucleotide) regulatory elements of translation of messenger RNAs to amino acids. Extracellular circulating microRNAs found in serum and plasma are easily measured in blood samples and are potential biomarkers for risk for development of type 2 diabetes, showing changes in expression levels prior to the onset of impaired glucose metabolism. (4,5). Because microRNAs capture both underlying genetic risk and responses to behavioral factors (e.g. diet and physical activity) (6,7) they represent several potential biomarker applications. First, as prognostic biomarkers, (8) there is the possibility for improved granularity in detecting glycaemic progression prior to the onset of type 2 diabetes. Second, because microRNAs are associated with individual pathways that singularly and collectively may lead to type 2 diabetes, they are potentially diagnostic biomarkers (8) that will provide greater understanding of which pathways are activated in one individual. Circulating microRNAs are also associated with complications (e.g. endothelial dysfunction) from impaired glucose metabolism that precedes and characterizes type 2 diabetes. (9,10)

Prior studies that evaluated relationships between circulating microRNAs and risk for type 2 diabetes were primarily limited to single measurements of microRNAs. The cross-sectional design of prior studies limits determination of which microRNAs are potentially prognostic biomarkers associated with trajectories of glucose metabolism as opposed to complications from impaired glucose metabolism. (11,12) The goals of this pilot study were to generate preliminary data about microRNAs as prognostic biomarkers by studying relationships between the trajectories of individual microRNAs and trajectories of fasting blood glucose over time. In addition, associations between risk factors (i.e. weight and fasting blood glucose) for type 2 diabetes following a behavioral intervention trial and circulating microRNAs are presented.

Research design and methods

Study participants

The previously completed Practicing Restorative Yoga vs. Stretching for the Metabolic Syndrome (PRYSMS) study (clinicaltrials.gov identifier NCT01024816) evaluated the effects of restorative yoga versus active stretching on blood glucose in community dwelling adults at risk for type 2 diabetes. (13) Participants in PRYSMS were recruited from the San Francisco and San Diego areas and met the International Diabetes Federation criteria for the metabolic syndrome. (14) Randomization was stratified by sex and race/ethnicity. Participants (n = 171) were randomized to either a restorative yoga or active stretching intervention for 12 months. Both interventions were delivered in a group setting twice weekly for 12 weeks and then weekly for 12 weeks and then monthly for 24 weeks. Participants were asked to practice yoga or stretching at home for at least 30 min three times per week. Fasting blood glucose was measured at baseline and every 3 months for the duration of the 1-year intervention. Plasma was isolated from whole blood and stored at −80°C.

Data from a recently completed randomized controlled trial of a weight loss intervention in Filipino Americans (Fit and Trim Trial) are also presented. The Fit and Trim study was a randomized controlled trial of a physical activity and diet lifestyle intervention for Filipino-Americans. The purpose of the trial was to evaluate an adaptation of the diabetes prevention program (15) that uses mobile-persuasive technologies, cultural tailoring for Filipino-Americans and a social networking component. Over 3 months, the program's goals were for the participants to lose 5% body weight, increase to and maintain 12,000 steps per day, reduce total fat intake to 25% of daily calories and reduce sugar-sweetened beverages to one every 2 weeks. A total of 62 participants, recruited from the San Francisco Bay Area using community outreach strategies, completed the study. In this study, 3-h oral glucose tolerance tests were performed at baseline. After 3 months, fasting blood glucose and insulin were measured. At both time points, plasma was isolated from whole blood and stored at −80°C. We studied a random subset (n = 8) of participants pre-intervention and 3-months post-intervention.

MicroRNA quantitation

The Firefly Bioworks Multiplex Circulating MicroRNA Assay (Abcam Fireplex, Cambridge, MA, USA) was used to quantify 353 microRNAs from plasma at four time points in a random subset of participants (n = 10) stratified by sex and intervention group from the PRYSMS study. The same assay was used to measure microRNAs from plasma from an additional random subset of participants (n = 8) stratified by sex and intervention group from the Fit and Trim study. This assay performs direct detection of microRNAs from plasma without an isolation step.

Statistical analysis

Descriptive statistics were calculated using means and standard deviations to evaluate demographic and clinical characteristics of participants in the PRYSMS and Fit and

Trim studies and comparisons between groups used Student's t-test (Stata version 13, College Station, TX).

The secondary analysis of the PRYSMS study focused on changes in fasting blood glucose over time. In order to identify patterns of fasting blood glucose in the PRYSMS sample (n = 171) (Figure 1), growth mixture modelling (GMM) was performed. GMM is an extension of latent growth curve analysis that extends the estimation of a single growth curve – represented as latent variables: intercept and slope coefficients and variance components for them – to the estimation of a new latent categorical variable that identifies latent growth curves for two or more classes. (16–19) The model fit for the GMM was assessed statistically by identifying the model with the lowest Bayesian Information Criterion and by testing the 'K' versus 'K-1' class models to determine whether a model with K classes fit the data better than a model with K-1 classes with the Vuong–Lo–Mendell–Rubin likelihood ratio test. (17,20,21) Two phenotypic groups (i.e. latent classes) from five glucose measurements over 1 year were identified (Figure 2, panel A) (Mplus Version 7.4). One GMM group exhibited variable blood glucose and the other group stable blood glucose. The study described here included a subset (n = 10) of participants randomly selected with equal distribution between the GMM groups and across the restorative yoga and active stretching groups. Trajectories of fasting blood glucose for this subset of participants are shown in Figure 2, panel B.

Expression of individual microRNAs was normalized using the global geometric mean signal of all reliably detected microRNAs in the assay.

Repeated measures analysis of variance was used to determine which microRNAs changed over time between the variable and stable glucose groups in the PRYSMS study (Stata v 14.2, College Station, TX). The false discovery rate method (22) at a threshold of 0.1 was used to adjust the significance levels and identify significant

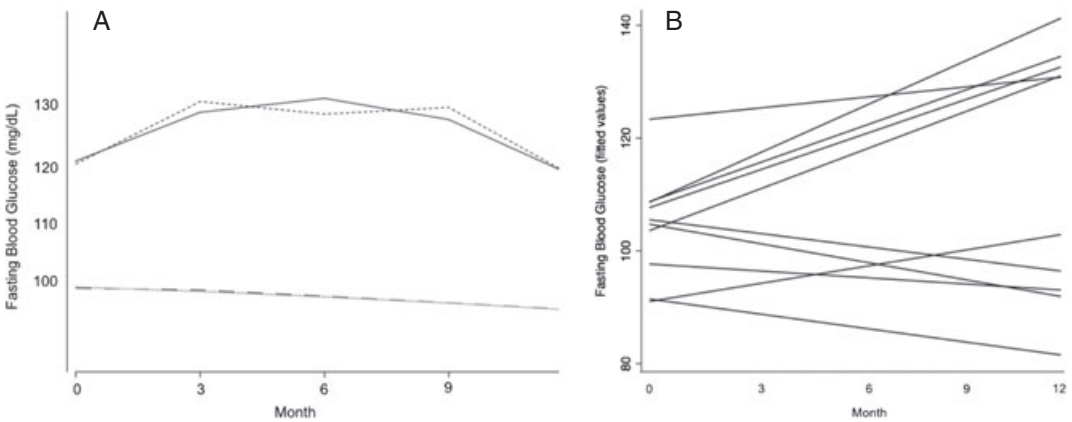

Figure 1 Panel A shows the predicted and actual trajectories of fasting blood glucose in the variable (short dashed line and solid black line, respectively) and stable (long dashed line and solid grey line, respectively) growth mixture modeling classes over 12 months. Panel B shows the fitted trajectories of fasting blood glucose for individual participants included in the study.

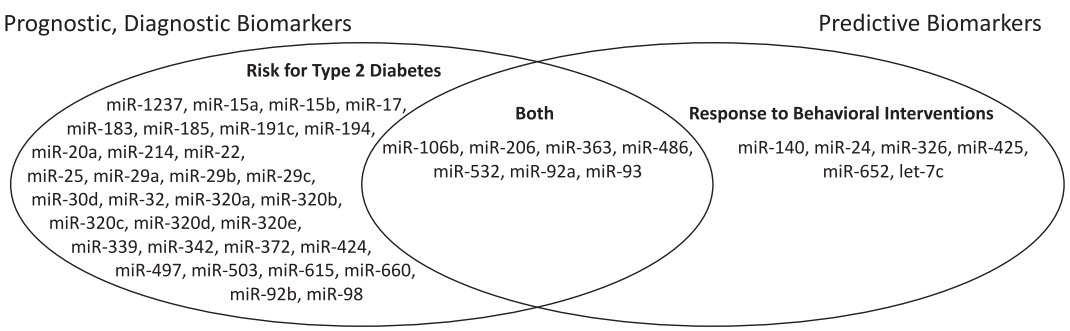

Figure 2 MicroRNAs (miRs) associated with risk for type 2 diabetes were identified in the Practicing Restorative Yoga vs. Stretching for the Metabolic Syndrome trial. MicroRNAs associated with response to a behavioral intervention were identified in the Fit and Trim weight loss trial. All microRNAs shown were measured in both studies.

changes over time. Linear models were used to determine whether the trajectories of individual microRNAs were statistically significantly positive or negative or were variable compared with trajectories of fasting blood glucose.

In the Fit and Trim study, paired Student's t-tests were used to compare differences in individual microRNAs before and after the 3-month physical activity and dietary intervention. Pearson's correlation coefficients were calculated to determine associations between baseline levels of individual microRNAs and weight change after 3 months and between changes in individual microRNAs and weight change over 3 months.

Results

The subset of participants from the PRYSMS trial included in this study was equally distributed by sex and had a mean age of 57 ± 4 years. Clinical characteristics at baseline between the two glucose classes are shown in Table 1. Overall, the variable blood glucose group had a mean increase in fasting blood glucose after 12 months of 2.2 mg dL^{-1} compared with no change in the stable blood glucose class. Forty-five individual microRNAs were differentially expressed between the GMM groups over 1 year ($p < 0.05$) (Table 2). Of these, the overall trajectories were positive for one microRNA (i.e. miR-320c) and negative for three microRNAs (i.e. miR-22, miR-372 and miR-339) in the variable glucose group and negative for one microRNA (i.e. miR-423) in the stable glucose group. For the remaining statistically significant microRNAs, the trajectories were variable (i.e. polynomial) across the five time points.

Clinical characteristics of the Fit and Trim subset of participants, included in this study, are shown in Table 3. The mean weight loss after the intervention was 9 ± 4 lb. This represented a 4.8% weight loss following the intervention. In these eight participants, a total of 13 microRNAs were differentially expressed after the weight loss intervention ($p < 0.1$). Four (i.e. miR-326, miR-24, miR-425 and miR-652) exhibited increased expression after the 3-month intervention. The remaining nine (i.e. miR-106b, miR-140, miR-20b, miR-363, miR-486, miR-532, miR-92a, miR-93 and miR-let7c) exhibited decreased expression after the intervention. Seven

Table 1 Clinical characteristics of participants in the Practicing Restorative Yoga vs. Stretching for the Metabolic Syndrome trial ($n = 10$).

	Stable blood glucose class	Variable blood glucose class	*p*-value
Body mass index (kg m^{-2})	32.6 ± 6.7	35.0 ± 5.5	0.39
Weight (lb)	205 ± 41	224 ± 31	0.26
Total cholesterol (mg dL^{-1})	218 ± 39	193 ± 38	0.16
LDL-c (mg dL^{-1})	131 ± 34	108 ± 33	0.14
HDL-c (mg dL^{-1})	53 ± 9	47 ± 14	0.27
Triglycerides (mg dL^{-1})	167 ± 70	187 ± 90	0.63
Fasting blood glucose (mg dL^{-1})	95 ± 8	117 ± 11	<0.001
Hemoglobin A1c (%)	5.6 ± 0.4	6.5 ± 0.6	<0.05

HDL-c, high-density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol.

Table 2 MicroRNAs differentially expressed between the variable and stable blood glucose groups in the practicing Restorative Yoga vs. Stretching for the Metabolic Syndrome trial

MicroRNA	*p*-value
miR-20a-5p	<0.001
miR-22-3p	<0.001
miR-320c	<0.001
miR-320e	<0.001
miR-92a-3p	<0.001
miR-93-3p	<0.001
miR-17-5p	0.001
miR-320a	0.001
miR-320b	0.001
miR-342-3p	0.001
miR-497-5p	0.001
miR-93-5p	0.001
miR-15b-3p	0.003
miR-185-5p	0.003
miR-183-3p	0.004
miR-423-5p	0.005
let-7b-5p	0.007
miR-20b-5p	0.007
miR-29a-3p	0.007
miR-106b-5p	0.009
miR-15a-5p	0.009
miR-214-3p	0.011
miR-29b-3p	0.011
miR-106a-5p	0.014
miR-194-5p	0.017
miR-320d	0.017
let-7g-5p	0.020
miR-424-5p	0.021
miR-660-5p	0.024
let-7i-5p	0.026
miR-363-3p	0.026
miR-92b-3p	0.027
miR-181c-3p	0.028
miR-532-3p	0.029
miR-1237-3p	0.030
miR-32-5p	0.031
miR-372-3p	0.031
miR-98-5p	0.031
miR-29c-3p	0.032
miR-503-5p	0.032
miR-30d-5p	0.035
miR-615-5p	0.040
miR-25-3p	0.042
miR-339-3p	0.046

Table 3 Clinical characteristics of participants in the Fit and Trim trial (n = 8).

	Baseline	3 Months	Change
Body mass index (kg m^{-2})	30.5 ± 2.8	28.9 ± 3.4	−1.5 ± 0.8
Weight (lb)	180 ± 32	172 ± 35	−9 ± 4
Total cholesterol (mg dL^{-1})	187 ± 31	173 ± 31	−14 ± 31
LDL-c (mg dL^{-1})	103 ± 20	97 ± 22	−5 ± 10
HDL-c (mg dL^{-1})	48 ± 12	49 ± 9	1 ± 6
Triglycerides (mg dL^{-1})	208 ± 176	155 ± 150	−55 ± 186
Fasting blood glucose (mg dL^{-1})	85 ± 4	83 ± 5	−2 ± 3
T120 blood glucose (mg dL^{-1})	109 ± 37	n/a	n/a
Hemoglobin A1c (%)	5.6 ± 0.2	5.5 ± 0.2	−0.1 ± 0.2
Fasting insulin (μIU mL^{-1})	4.8 ± 3.5	5.6 ± 1.9	0.8 ± 2.2

HDL-c, high-density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; n/a, not assessed.

microRNAs overlapped with the PRYSMS findings (i.e. miR-106b [fold change 0.73, $p < 0.1$], miR-20b [fold change 0.80, $p < 0.05$], miR-363 [fold change 0.66, $p < 0.1$], miR-486 [fold change 0.72, $p < 0.1$], miR-532 [fold change 0.75, $p < 0.05$], miR-92a [fold change 0.81, $p < 0.1$] and miR-93 [fold change 0.84, $p < 0.1$]) (Figure 1). Changes in levels of three microRNAs (i.e. miR-146a, miR-151a and miR-23a levels) were very strongly positively correlated ($r^2 > 0.8$, $p < 0.05$) with changes in weight over 3 months. An additional nine microRNAs (i.e. miR-181b, miR-181d, miR-21, miR-221, miR-222, miR-223, miR-23, miR-24 and miR-27b) were strongly positively correlated ($0.6 < r^2 < 0.8$, $p < 0.05$) with weight change. Baseline levels of eight microRNAs (i.e. miR-143, miR-145, miR-146, miR-191, miR-221, miR-23a, miR-29a and miR-584) have a positive association with weight change after 3 months ($r^2 > 0.6$, $p < 0.05$). There were no negative associations between baseline levels of individual microRNAs or change in levels of individual microRNAs with weight change over 3 months.

Conclusion

MicroRNAs are emerging as possible prognostic biomarkers to identify individuals at high risk for type 2 diabetes, and diagnostic biomarkers to characterize individual differences in disease etiology. A limitation of prior studies is the inability to differentiate circulating microRNAs related to risk for type 2 diabetes versus complications of type 2 diabetes. In this study, numerous circulating microRNAs with trajectories that relate to trajectories of fasting blood glucose were identified.

Prior observational studies on microRNAs associated with risk for type 2 diabetes have been primarily cross-sectional or limited to two measures of fasting blood glucose or related markers. (11,12,23–25) A few prior studies included repeated measures of microRNAs that were pre-post interventions that target pathways associated with risk for type 2 diabetes (e.g. exercise). (6,7,24) One of the early contributions in this field identified microRNAs associated with prevalent type 2 diabetes after 10 years in a large cohort study of Europeans. (23) Among the microRNAs identified was miR-126, which is now well established to be an endothelial-related microRNA (26) and possibly related to complications from type 2 diabetes as opposed to mechanisms underlying development of the primary disease (9,10,27). There are potentially useful biomarker applications for microRNAs related to complications, but these are discrete from applications for microRNAs associated with prodromal identification of risk for type 2 diabetes.

This pilot study provided preliminary evidence for 45 potential prognostic microRNA biomarkers with trajectories over 1 year that associate with trajectories of fasting blood glucose. Among these are numerous microRNAs that were previously identified in our own and other prior studies. For example, miR-106b, miR-17, miR-20a and miR-93 are related to a measure of insulin resistance, (28) miR-214, miR-22 and miR-320a are related to insulin resistance and responses to thiazolidenidones (4) and miR-15a, miR-320a and miR-423 are related to glycaemic impairment and progression (5). The latter two, miR-320a and miR-423, have also been reported in other studies with related mechanisms. (29,30) A future potential application of microRNAs is as predictive biomarkers (8) to optimize risk reduction interventions. Preliminary work from our group identifies microRNAs that are also associated with behavioral interventions like weight loss in response to a physical activity and diet intervention (e.g. Fit and Trim trial presented as confirmatory data). Taken together, these are promising preliminary data to show that microRNAs are antecedent markers for risk for type 2 diabetes before the development of impaired glucose metabolism and type 2 diabetes (i.e. prognostic and diagnostic biomarkers) and microRNAs may be useful for predicting responses to interventions (i.e. predictive biomarkers). A summary of preliminary data shows some microRNAs show overlap as prognostic and diagnostic biomarkers for risk for type 2 diabetes and predictive biomarkers for responses to interventions, while others are unique (Figure 1).

This study provided preliminary data from a hypothesis generating pilot study. Future studies are needed in three key areas. The first is to validate prognostic biomarker applications of microRNAs in larger samples and to investigate whether clusters of related microRNAs might enhance risk prediction. Second is to further investigate microRNAs as diagnostic biomarkers by identification of

mechanistic targets of microRNAs so that underlying pathways can be identified. This will facilitate improved risk stratification and targeted treatments. Third is to expand on preliminary research on microRNAs as predictive biomarkers that predict responses to risk reduction interventions in order to optimize treatment strategies for individuals.

This preliminary study has limitations, including a small sample size and moderate differences in fasting blood glucose between groups. Despite these limitations, a large number of microRNAs that were significantly associated with trajectories of fasting blood glucose after adjusting for multiple comparisons were identified. These findings support the need for future studies in larger samples sizes with greater power to model clusters of microRNAs. MicroRNAs may become useful prognostic, diagnostic and predictive biomarkers for prevention and treatment of type 2 diabetes.

Author Contributions

E. F. performed data collection and data analysis and had primary responsibility for manuscript preparation. A. M. conducted the PRYSMS study, provided biospecimens and contributed to data analysis and manuscript preparation. Y. F. completed the Fit and Trim study and contributed to manuscript preparation. B. C. and I. E. A. performed data analysis and contributed to manuscript preparation. B. E. A. consulted on data collection and data analysis and contributed to manuscript preparation.

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